Allatostatin-A neurons inhibit feeding behavior in adult *Drosophila*

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How the brain translates changes in internal metabolic state or perceived food quality into alterations in feeding behavior remains poorly understood. Studies in *Drosophila* larvae have yielded information about neuropeptides and circuits that promote feeding, but a peptidergic neuron subset whose activation inhibits feeding in adult flies, without promoting metabolic changes that mimic the state of satiety, has not been identified. Using genetically based manipulations of neuronal activity, we show that activation of neurons (or neuroendocrine cells) expressing the neuropeptide allatostatin A (AstA) inhibits or limits several starvation-induced changes in feeding behavior in adult *Drosophila*, including increased food intake and enhanced behavioral responsiveness to sugar. Importantly, these effects on feeding behavior are observed in the absence of any measurable effects on metabolism or energy reserves, suggesting that AstA neuron activation is likely a consequence, not a cause, of metabolic changes that induce the state of satiety. These data suggest that activation of AstA-expressing neurons promotes food aversion and/or exerts an inhibitory influence on the motivation to feed and implicate these neurons and their associated circuitry in the mechanisms that translate the state of satiety into alterations in feeding behavior.

homeostasis | satiation | foraging | proboscis extension reflex | neuromodulation

To maintain energy homeostasis, the brain translates changes in internal metabolic state into alterations in feeding behavior. Understanding how this translation occurs at the molecular and neural circuit levels is of fundamental importance both as a general model for state-dependent modification of behavior and also for its relevance to human obesity and associated diabetic and cardiovascular disease (1). *Drosophila melanogaster* provides an excellent model system to study feeding behavior (2, 3) due to its powerful genetics, and because many elements of metabolic homeostasis are conserved between flies and mammals (2, 4–6).

Most studies of feeding in *Drosophila* have emphasized mechanisms that promote food intake, primarily in larvae (reviewed in refs. 4, 7, 8). Neuropeptides have been a central focus because of their neuromodulatory role, genetic accessibility, and relevance to feeding and satiety in mammals (9–15; reviewed in refs. 4, 8, 16, 17). In larvae, neuropeptide F (NPF) and its receptor, NPF-R, have been shown to promote feeding (11). NPF and related peptides also mediate the influence of food deprivation on several behaviors in adult flies (10, 18). Other neuropeptides, such as adipokinetic hormone (AKH) have been shown to influence feeding-related behaviors as well (19, 20). However, because neuropeptides are often involved in metabolic homeostasis (21), which in turn regulates feeding behavior, it can be difficult to determine whether a given neuropeptide regulates feeding behavior directly or indirectly via metabolic influences.

The neurobiological mechanisms that inhibit or limit feeding behavior, especially in adult *Drosophila*, are less well understood. Experiments in blowflies have indicated that a primary mechanism that promotes satiety in that species is inhibitory proprioceptive feedback from the foregut and crop (reviewed in refs. 22, 23), but whether such a mechanism also operates in *Drosophila* is unclear. In *Drosophila* larvae, NPF-R–expressing neurons are negatively regulated by the insulin-like peptides (DILPs) (13, 24). The neuropeptide hugin has been shown to inhibit feeding during the transition to a novel food resource (12), whereas leucokinin has been shown to negatively regulate meal size (15). However, a peptidergic neuron subset whose activation inhibits feeding in adult flies, without promoting metabolic changes that mimic the state of satiety, has not been reported.

In other insect species, allatoregulatory peptides, including allatostatins A, B, C and the allatotropins, have been implicated in the regulation of feeding (reviewed in refs. 16, 17). In vivo injection of allatostatin A (AstA) has been shown to suppress food intake in the cockroach (25, 26), whereas ex vivo experiments have suggested a role to inhibit gut motility (27). On the basis of these data, it has been suggested that the inhibitory effect of AstA peptide on feeding likely reflects its myoinhibitory influence (16). However, the influence of AstA on feeding may also reflect a role for this peptide in the CNS (25, 28). Indeed, in many insect species, AstA acts centrally to inhibit the synthesis of juvenile hormone (JH), which promotes feeding (25, 28). Injection of AstA RNAi produced a biphasic effect on feeding in virgin female crickets (26), perhaps via effects on JH. Although AstA peptide injection inhibited feeding in some insect species where it does not inhibit JH synthesis (16), metabolic effects of this manipulation were not excluded. Thus, the physiological mechanism of action of AstA is not clear. There are no studies examining the effect on feeding behavior of manipulating AstA-expressing neurons, and it is the neurons, not the peptide, whose activity is regulated under normal physiological circumstances.

Drosophila offers genetic tools to investigate the role of AstA-expressing neurons, which are not available in other insect species (29). The expression of AstA in *Drosophila* suggests that this neuropeptide may play a role in the regulation of food intake in this species (30), but direct evidence for such a function is lacking. Here we have gained genetic access to a small subpopulation of AstA-expressing neurons, and have investigated their function using genetic tools to manipulate their activity. Our data suggest that these AstA neurons are part of a circuit that negatively regulates feeding behavior, which acts downstream of metabolic changes that underlie the state of satiety.

Results

***AstA–GAL4* Transgenic Flies Express *GAL4* in a Subset of AstA Neurons.** We constructed *AstA* promoter–*GAL4* transgenic flies that contain 2.1 kb upstream of the predicted transcription start

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site of the *AstA* gene (Fig. 1*A*). We characterized the expression pattern of this *GAL4* driver using *UAS-mCD8::GFP* (31), a membrane-tethered fluorescent reporter, and counterstained with a monoclonal antibody raised against *Diploptera punctata* AstA (28) (previously used to characterize AstA expression in *Drosophila*) (30).

This analysis revealed that *AstA–GAL4* is exclusively expressed in a subset of AstA peptide-expressing neurons: in six central brain-, ~30 optic lobe-, three ventral nerve cord (VNC)-, and two peripheral-AstA–expressing neurons per hemisection (Fig. 1 *B–J* and Fig. S1 *A–F*). Double-labeled (GFP^+ and $AstA^+$) fibers were observed to innervate the subesophageal ganglion (SOG), protocerebrum, and pars intercerebralis (Fig. 1 *B–G*). Double-labeled neurons in the dorsal VNC (Fig. 1 *H–J*, *Inset*) send projections dorsally to innervate the lower midgut, the hindgut, and the rectum (Fig. S1 *G–L*). Coexpression of GFP/AstA was also seen near the midgut transitions and in neuroendocrine cells in the lower midgut (Fig. S1 *G–I* and *M–R*). No expression of the mCD8–GFP reporter was detected in $AstA^-$ cells.

AstA Neurons Inhibit Starvation-Induced Feeding Behavior. To study the role of AstA neurons in feeding behavior, we developed an assay in which such behavior is sensitive to the influence of food deprivation (32) (*Materials and Methods* and Fig. S2 *A–D*). To quantify the fraction of flies feeding, we added a neutral dye to their food and measured the proportion of flies with visually detectable dye in their gut (3, 33). Comparison with direct measurements of the volume of food intake, using colorimetric quantification of the dye in fly homogenates, indicated that this visual method of scoring was reliable above a threshold volume of ~15 nL food per fly. This volume is well below the average meal size of wild-type flies (3, 34) (Fig. S1*D*).

To activate AstA neurons, we expressed NaChBac, a bacterially derived, low threshold voltage-gated sodium channel that has been shown to increase neuronal excitability in *Drosophila* (35). Expression of NaChBac in *AstA–GAL4*-expressing neurons significantly reduced the fraction of starved flies that fed whether scored visually (Fig. 2*A*) or using colorimetric quantification (Fig. S2*E*). A reduction in feeding behavior was observed using either of two independent *AstA–GAL4* lines (Fig. 2A) and was also seen using an independent assay of feeding, the capillary feeding (CAFE) assay (34) (Fig. S2*F*). Inhibition of feeding was also observed in unstarved *AstA/NaChBac* flies, although the magnitude of the effect depended on assay conditions and the strength of the *AstA–GAL4* driver (Fig. S2*G* and Footnote S1).

To confirm that this phenotype was indeed due to hyperactivation of AstA neurons, we asked whether it could be rescued by simultaneously coexpressing an inwardly rectifying potassium channel, Kir2.1, which decreases neuronal excitability (36). Coexpression of Kir2.1 together with NaChBac in AstA neurons reversed the suppression of starvation-induced feeding

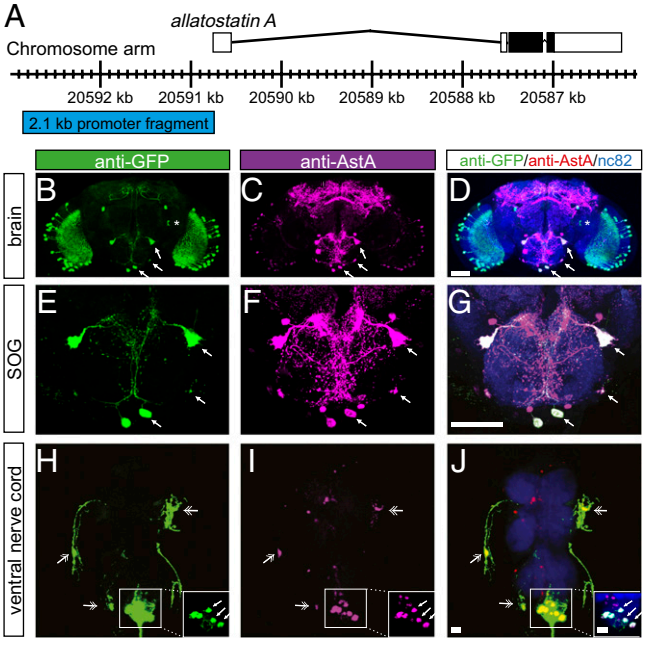

Fig. 1. *AstA–GAL4* expression in the adult CNS. (*A*) Structure of *Drosophila AstA* gene and origin of upstream sequence in *AstA–GAL4* transgenic flies (blue box). Black boxes are coding exons. (*B–J*) Adult brain (*B–G*) and ventral nerve cord (*H–J*) from $AstA^1$*–GAL4*; *UAS–GFP* flies double immunostained using antibodies to GFP (*B*, *E*, and *H*) and *Diploptera* AstA (*C*, *F*, and *I*) (30). (*D*, *G*, and *J*) Merged images, counterstained with antibody to the neuropil marker nc82. Arrows in *B–G* indicate GFP/AstA double-positive cells. Asterisks in *B* and *D* indicate staining artifact. Double arrowheads in *H–J* indicate fibers exiting the VNC, imaged at a high gain setting to highlight nerve fibers. *Insets* show the boxed region, imaged at a lower gain setting to highlight GFP/AstA double-positive cell bodies in the abdominal ganglion (arrows). Higher resolution images of double-positive cells in the protocerebrum and optic lobe are shown in Fig. S1 *A–F*. (Scale bars, 50 μm.)

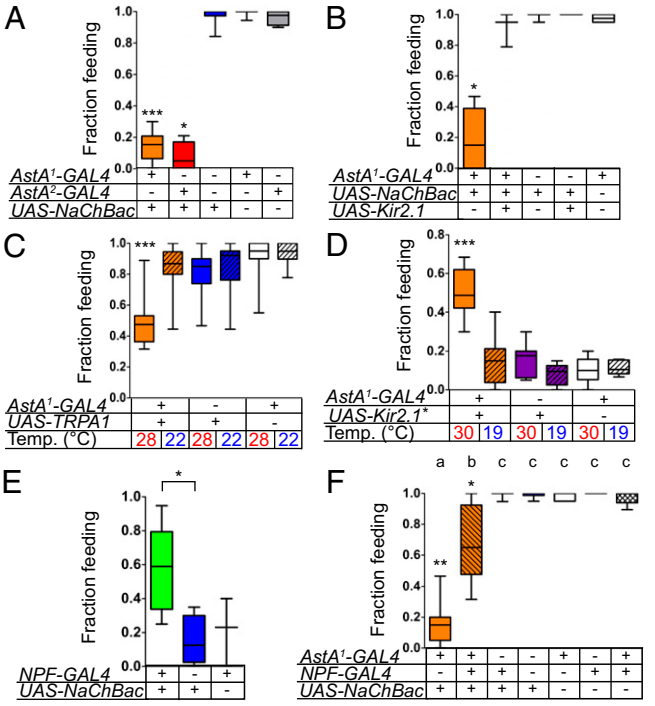

Fig. 2. Activation of AstA neurons inhibits feeding. (*A–F*) "Fraction feeding" represents average fraction of flies feeding within 1 h, following 24 h of starvation (*Materials and Methods*). Genotypes and temperatures (*C* and *D*) are indicated below bars; $AstA^1$ and $AstA^2$ represent two independent transgene insertions. Temperatures in *C* refer to assay conditions. In *D*, flies were raised at 19 °C and the experimental group shifted to 30 °C for 48 h before testing at 25 °C. Control genotypes showed no effect of temperature ($P = 0.7202$). Food source was 10 mM sucrose (*A–C* and *F*) or 100 mM sucrose plus 500 mM NaCl (*D* and *E*). Boxes and whiskers represent the quartile, minimum, and maximum values. In this and in all other experiments, unless otherwise indicated, *n* refers to the number of independent groups of 20 flies per genotype tested. Here *n* = (*A*) 4–20, (*B*) 3–7, (*C*) 12–15, (*D*) 7–16, (*E*) 3–8, and (*F*) 6–11. Statistical significance was calculated using Kruskal–Wallis one-way ANOVA with Dunn's correction (*A*, *B*, *E*, and *F*) or two-way ANOVA with Bonferroni correction (*C* and *D*), where $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$. Different letters above bars in *F* indicate genotypes that are significantly different from one another.

seen in *AstA/NaChBac* flies (Fig. 2*B*). This rescue suggests that the reduced feeding phenotype of *AstA/NaChBac* flies was indeed due to increased activity of AstA neurons.

To address the possibility that the *AstA/NaChBac* phenotype was due to developmental defects from constitutively activating neurons, we used *UAS–TRPA1* (37), a warmth-activated cation channel, to acutely activate AstA neurons in adult flies. When *AstA–GAL4*; *UAS–TRPA1* flies (raised at 22 °C) were shifted to the permissive temperature (28 °C) an hour before testing, significantly fewer flies fed, compared with genetic controls tested at the same temperature (Fig. 2*C*). In contrast, the feeding behavior of *AstA/TRPA1* flies tested at 22 °C did not differ from that of controls. Thus, the effect of TRPA1 was both temperature and genotype dependent and phenocopied the feeding phenotype observed using NaChBac. The penetrance of the TRPA1 phenotype, however, was weaker than that obtained using NaChBac, which may reflect a difference in the potency of these two effectors (29). Taken together, these data indicate that activation of *AstA–GAL4*-expressing neurons can suppress starvation-induced increases in feeding in adult flies.

To validate further the physiological relevance of AstA neuronal activity to feeding behavior, we next asked whether inhibition of these neurons would enhance food intake, using Kir2.1, together with tub-GAL80^{ts} (38) to restrict the expression of this potassium channel to adult flies. Because we predicted an increase in feeding as a consequence of Kir2.1 expression, and because control flies already show maximal levels of feeding in our standard assay, we reduced the level of baseline food intake by combining 100 mM sucrose with 500 mM NaCl. Under such conditions, <20% of control flies fed (Fig. 2*D*). Induction of *UAS–Kir2.1* expression, by inactivation of Gal80^{ts} at 30 °C, significantly increased the fraction of flies that fed in this assay, compared with both genetic controls at the same temperature, as well as to the same genotype at the nonpermissive temperature of 19 °C (Fig. 2*D*). Both genetic and temperature controls showed statistically equivalent levels of feeding. These data indicate that inhibition of AstA neuronal activity can increase feeding. Taken together, these loss- and gain-of-function data suggest that AstA neurons play a physiologically relevant role to inhibit or limit feeding in adult *Drosophila*, in the setting of either food deprivation or diminished food quality.

NPF Neuron Activation Suppresses the Inhibitory Influence of AstA Neuron Activation on Feeding. Hyperactivation of AstA neurons could suppress food intake by inhibiting pathways that normally promote feeding, or it could exert this influence more indirectly (for example, by making the flies "sick"). As a first step toward distinguishing these possibilities, we asked whether the effects of AstA neuron activation could be overridden by simultaneous activation of a pathway implicated in the promotion of feeding. In larvae, NPF overexpression increases foraging behavior and the acceptance of noxious food sources (11), consistent with a role in promoting a hunger-like state. In adult flies, NPF signaling is necessary and sufficient to elicit hunger-mediated associative learning (18). Consistent with these findings, we found that activation of NPF neurons significantly increased feeding (Fig. 2*E*). Thus, activation of NPF vs. AstA neurons causes opposite effects on feeding in adult flies. In flies expressing *UAS–NaChBac* in both *AstA–GAL4* and *NPF–GAL4* neurons, the reduced feeding behavior caused by activation of AstA neurons alone was largely (although not completely) rescued (Fig. 2*F*). These epistasis data suggest that AstA neuron activation suppresses feeding through a mechanism that can be overridden or bypassed by simultaneously activating a pathway that promotes feeding in adults. This in turn argues that the influence of AstA neuron activation to suppress feeding is not nonspecific, but is exerted through an influence on pathways that normally promote food intake (Fig. 4*H*).

AstA Neuron Activation Does Not Promote Metabolic Changes Mimicking Satiety. We next investigated possible physiological mechanisms through which the activation of AstA neurons might suppress feeding. Because allatoregulatory peptides have been implicated in the control of gut motility (16, 17), we asked whether activating AstA neurons increased the content of food in the gut. Using food dye to measure gut content, we determined that *AstA/NaChBac* flies neither retained excess food in the gut after starvation nor differed from controls in their total gut content before food deprivation (Fig. 3*A*). These data argue against the idea that activation of AstA neurons inhibits feeding by blocking food excretion.

As mentioned earlier, in some insect species, allatostatins can exert metabolic influences, such as inhibition of JH synthesis or of enzyme release in the gut (16), which might indirectly influence feeding behavior. We therefore investigated whether the effect of AstA neuron activation on feeding might be due to metabolic changes that mimic the state of satiety. We first explored the possibility that *AstA/NaChBac* flies have excess energy reserves or decreased metabolism. We directly measured energy reserves in unstarved *AstA/NaChBac* flies by quantifying their mass and levels of triglycerides and glucose. However, there was no significant difference between *AstA/NaChBac* and control (*UAS–NaChBac/+*) flies in these parameters (Fig. S3 *A–C*). Furthermore, fly mass and triglyceride and glucose levels in *AstA/NaChBac* flies were depleted upon starvation to similar levels as those of control (*UAS–NaChBac/+*) flies (Fig. 3 *B–D*).

As an indirect, physiological measure of energy reserves, we also examined the starvation resistance of *AstA/NaChBac* flies. Genetic manipulations that diminish flies' energy reserves often reduce their survival time under starvation conditions (19), suggesting, conversely, that a manipulation that increased survival time under starvation conditions might reflect an increase in energy reserves. However, activation of AstA neurons did not enhance starvation resistance compared with control (*UAS–NaChBac/+*) flies, while the starvation resistance of *AstA-GAL4/+* flies was slightly but significantly higher (Fig. 3*E*). We also investigated whether activation of AstA neurons could override an experimental manipulation that sensitizes flies to starvation. Activation of neurons expressing the neuropeptide corazonin (Crz) (39) has been reported to reduce both starvation resistance and triglyceride (fat) stores (40), suggesting that it decreases energy reserves. Using an independently generated *Crz–GAL4* line, we confirmed that activation of *Crz–GAL4* neurons (using *UAS–NaChBac*) increased starvation sensitivity (Fig. S3*D*) and found that it also increased food intake under our assay conditions (Fig. S3*E*). Thus, activation of *Crz–GAL4* neurons, like that of NPF neurons, has an opposite effect on feeding than does activation of AstA neurons. However, in contrast to the results obtained with *NPF–GAL4*, coactivation of Crz neurons did not rescue the reduced food intake phenotype caused by activation of AstA neurons (Fig. S3*F*). Nevertheless, despite the fact that activation of AstA neurons still suppressed feeding under these conditions, it failed to rescue the reduced starvation resistance characteristic of *Crz/NaChBac* flies (Fig. S3*D*). These data provide further evidence against the idea that activation of AstA neurons reduces feeding indirectly, by increasing energy reserves.

Starvation promotes several types of behavioral adaptations in addition to increased food intake, including increased spontaneous locomotor activity (20, 41, 42). Consequently, another explanation for the reduced feeding of *AstA/NaChBac* flies is that they do not exhibit a normal starvation-induced increase in locomotor activity and therefore burn fewer calories during starvation. However, both the average total locomotor activity (Fig. 3*F*) and starvation-enhanced locomotion (Fig. 3*G*) of *AstA/NaChBac* flies were similar to that of genetic controls. These data argue against the idea that activation of AstA neurons inhibits feeding indirectly, by reducing energy expenditure during starvation.

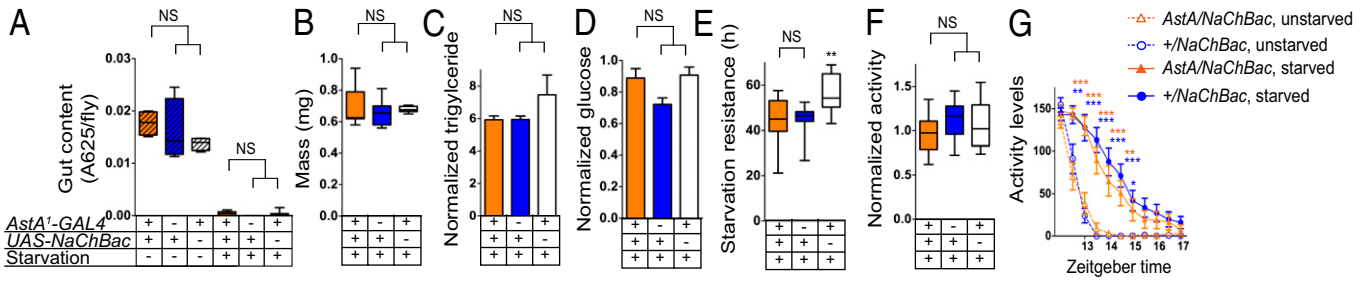

Fig. 3. Energy stores and expenditure are unaffected in starved *AstA/NaChBac* flies. (*A*) Gut content of unstarved and starved flies after feeding on dye-laced standard food for 2 d (n = 3–7). (*B*) Mass of starved flies (n = 9–15). (*C*) Whole-body triglyceride levels in starved flies normalized to protein levels (n = 3). (*D*) Whole-body glucose levels in starved flies normalized to protein levels (n = 3). (*E*–*G*) Activity (beam-crossing) measurements in single-fly activity monitors. (*E*) Starvation resistance, measured as the time of the last beam crossing (n = 15–32 flies tested per genotype). *AstA–GAL4* control flies exhibited a small but significant difference compared with *UAS–NaChBac/+* flies. (*F*) Total activity during wet starvation, normalized to survival time (n = 15–32). (*G*) Activity of starved and unstarved flies during the second evening peak (n = 16). Level of starvation-induced hyperactivity (asterisks) did not differ significantly between genotypes ($P > 0.44$). (*A*, *B*, *E*, and *F*) Boxes and whiskers represent the quartile, minimum, and maximum values and statistical significance was calculated using Kruskall–Wallis one-way ANOVA with Dunn's correction. Values represent mean ± SEM, and one-way (*C* and *D*) or two-way ANOVA (*G*) with Bonferroni correction was used. $P > 0.05$ (NS), $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Activation of AstA Neurons Inhibits Starvation-Induced Enhancement of PER Behavior. Food deprivation also causes an increased responsiveness to food cues (43–45). To test whether activation of AstA neurons altered behavioral responsiveness to food cues, we used the proboscis extension reflex (PER) assay (46), in which the retracted proboscis is stimulated with increasing concentrations of a sucrose solution. The probability of proboscis extension increases as the concentration of sucrose is increased and is enhanced as a result of starvation (45, 47). In unstarved flies, activation of AstA neurons did not impair the PER across a range of sucrose concentrations, (Fig. 4*A*, dotted lines).

Starvation enhances the sucrose responsiveness of the PER in wild-type *Drosophila* (43, 45), measured as a leftward shift of the PER vs. sucrose dose–response curve (Fig. 4*A*, dashed vs. solid blue lines). Strikingly, activation of AstA neurons largely blocked this starvation-induced behavioral change (Fig. 4*A*, yellow vs. blue solid lines). This effect can also be measured as a statistically significant increase in the mean acceptance threshold (MAT) for sucrose, the concentration at which a PER response is elicited in 50% of the flies (Fig. 4*B*).

We next investigated whether the PER phenotype of starved *AstA/NaChBac* flies reflects a reduction in gustatory discrimination or simply decreased sucrose responsiveness. To do this, we performed a taste discrimination test using two very low concentrations of sucrose. Flies exhibit a preference for 5 mM vs. 1 mM sucrose in a two-choice assay (48). The preference of starved *AstA/NaChBac* flies for 5 vs. 1 mM sucrose was not significantly different from that of genetic controls in this assay (Fig. 4*C*), although the fraction of *AstA/NaChBac* flies that consumed any sucrose at all was significantly reduced, as expected (Fig. S4*B*). These data suggest that the PER phenotype of starved *AstA/NaChBac* flies is unlikely to reflect a reduced capacity for gustatory discrimination, but rather represents a decreased responsiveness to sucrose. Interestingly, in the SOG, the fibers of *AstA–GAL4* neurons exhibited varicosities in close association with those of GR5a gustatory neurons, which detect sucrose (49–51) and drive the PER (52, 53) (Fig. 4*E*).

The PER phenotype of *AstA/NaChBac* flies raised the possibility that the inhibition of poststarvation food intake in these flies was an indirect consequence of their decreased sucrose

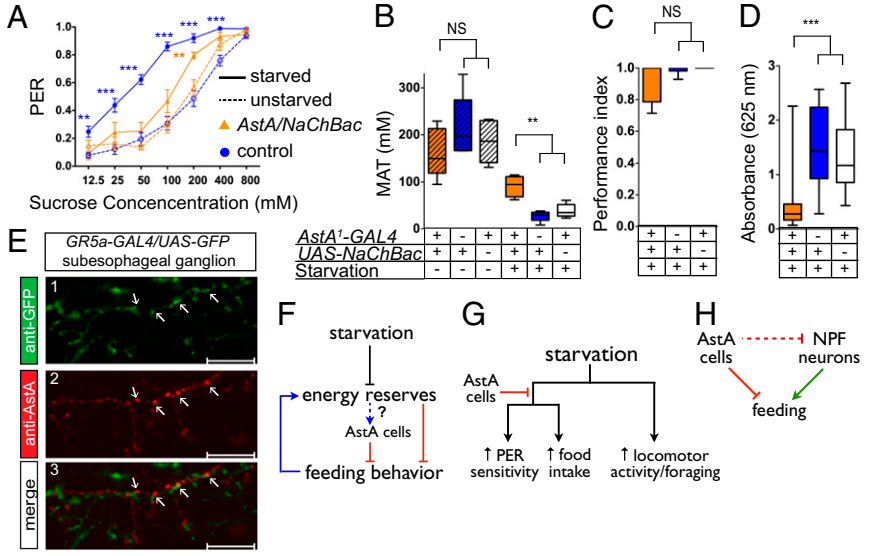

Fig. 4. Effect of AstA neuron activation on PER behavior. (*A*) Average (± SEM) fraction of unstarved (dotted lines) and 24-h wet-starved flies (solid lines) exhibiting a PER to the indicated concentrations of sucrose (n = 5–6, 10–20 flies per group). Responses of control genotypes are pooled (blue lines, see Fig. S4*A* for unpooled data). Asterisks in *A* denote statistically significant differences between starved vs. unstarved flies. (*B*) Mean acceptance threshold values for the indicated genotypes, starved or unstarved. MATs were interpolated from nonlinear regression analysis of data in *A*. (*C*) Performance in a sucrose-preference assay (*Materials and Methods*) (NS, $P = 0.3922$; n = 4). (*D*) Average volume of 800 mM sucrose consumed by starved individual flies hand-fed to satiation (n = 17–19 flies per genotype). Boxes and whiskers in *B*–*D* represent the quartile, minimum, and maximum values. Statistical significance was calculated using two-way ANOVA with Bonferroni correction (*A*) or Kruskall–Wallis one-way ANOVA with Dunn's correction (*B*–*D*), where $P > 0.05$ (NS), $**P < 0.01$, and $***P < 0.001$. (*E*) Fibers in the SOG of *GR5a-GAL4/UAS-mCD8::GFP* flies double immunostained with anti-GFP (1), anti-AstA (2), and overlay shown in (3). (Scale bar, 20 μm.) (*F*) AstA cells inhibit starvation-induced changes in feeding behavior downstream of (or parallel to) metabolic influences. (*G*) AstA cells inhibit starvation-induced increases in food intake and PER sensitivity, but not locomotor activity. (*H*) Antagonistic control of feeding by AstA and NPF neurons.

responsiveness. Alternatively, activation of AstA neurons could exert independent influences on these two behaviors. As a first step toward distinguishing these alternatives, we measured the total volume of food consumed by flies hand-fed 800 mM sucrose, a concentration at which 100% of both *AstA*/*NaChBac* and control flies exhibited a PER response (Fig. 4*A*). Strikingly, activation of AstA neurons significantly inhibited the intake of 800 mM sucrose, compared with controls (Fig. 4*D*). The reduced food intake observed under these conditions therefore cannot be explained by impaired PER behavior. The observation that AstA neuron activation impairs food intake in hand-fed flies also indicates that the reduced feeding seen in freely moving animals (Fig. 2) is not due to impairments in vision, olfaction, or other functions necessary for food localization.

Discussion

The problem of how neural circuits that control feeding are regulated by metabolism is a fundamental one, whose logic can be studied in model organisms independently of whether the particular gene products or neural circuits involved have direct mammalian homologs. To approach this question, it is necessary to define circuits that directly control feeding behavior, which may serve as targets of metabolic influence. Here we demonstrate that AstA neurons exert an inhibitory influence on multiple aspects of feeding behavior in *Drosophila* (Fig. 4*G*). This influence can be observed in the absence of any detectable effects on metabolism or energy expenditure (Fig. 3 and Fig. S3), arguing that AstA neurons do not simply promote metabolic changes that mimic or cause satiety. This study therefore provides evidence of a neural circuit that depresses food intake, which is distinct from circuits that promote feeding (Fig. 4*H*) and acts downstream of metabolic influences (Fig. 4*F*).

Mechanism and Locus of AstA Neuron Action. The prevailing model for satiety in insects, based on studies in blowflies, is that proprioceptive feedback from foregut and crop distention is transmitted to the brain via the neck connective, thereby inhibiting central circuits that control feeding behavior (reviewed in refs. 22, 23). Although it is not known whether this mechanism operates in *Drosophila*, it raises the possibility that AstA neurons might inhibit feeding by regulating gut distention or proprioceptive feedback from the gut to the brain. However, we found no evidence that activation of AstA neurons increases gut volume. We did not observe expression of our *AstA–GAL4* drivers, or of AstA itself, in the foregut or crop (Fig. S1 *P–R* and ref. 30). Nevertheless, both AstA and our *AstA–GAL4* drivers are expressed in a subset of gut neuroendocrine cells (Fig. S1 *G–I* and ref. 30). Because these neuroendocrine cells express "pan-neuronal" drivers such as Elav, they cannot easily be manipulated independently of AstA neurons in the central brain and PNS. Therefore, we cannot exclude a role for AstA-expressing gut neuroendocrine cells in the control of feeding behavior.

AstA-expressing nerve fibers ramify within the SOG, where they exhibit varicosities in close proximity to the central projections of GR5a gustatory neurons (Fig. 4*E*), which detect sweet tastants (50, 51). Given that GR5a neurons control PER behavior (52, 53), and that activating AstA neurons prevents the starvation-induced enhancement of sucrose-evoked PER behavior, it is possible that AstA neurons act in the SOG, either directly on GR5a fibers or on other neuronal populations that arborize in this structure, to regulate the PER. These observations, and the fact that AstA neuron activation inhibits feeding but not PER behavior in starved flies hand-fed 800 mM sucrose (as well as in unstarved flies), suggest that the PER and food intake may be controlled by different populations of AstA neurons.

The genetic manipulations of AstA neuronal activity performed here are likely to affect the release of both AstA itself, as well as other cotransmitters. Presently, there are no loss-of-function alleles of either *AstA* or its putative receptors (54), and, in our hands, expression of RNAi's for these genes was ineffective at reducing transcript levels. Furthermore, we failed to observe any feeding-related phenotype upon injection of flies with AstA synthetic peptide. However, as noted earlier, injection of AstA peptide inhibits food intake in a number of other insect species (16). Furthermore, orthologs of AstA receptors have been shown to play a role in feeding in mammals (55–57) and *Caenorhabditis elegans* (58). Given these data, it is likely that AstA itself plays a role to promote satiety or aversion to unpalatable food resources in *Drosophila*, but this remains to be demonstrated.

Epistatic Interactions Between AstA and Other Peptidergic Neurons Controlling Feeding and Energy Metabolism. We performed genetic epistasis experiments to examine interactions between AstA neurons and other classes of peptidergic neurons implicated in the control of feeding. Simultaneous activation of NPF neurons and AstA neurons largely relieved the inhibition of feeding caused by activation of AstA neurons on their own. This suggests that NPF and AstA neurons may act antagonistically to control feeding (Fig. 4*H*). In contrast, coactivation of neurons expressing Crz failed to rescue the reduced feeding caused by AstA neuron activation, despite the fact that activation of Crz neurons on its own enhanced food intake. Thus, although both NPF and Crz neurons promote food intake, they exhibit opposite epistatic interactions with AstA neurons (Fig. 4*H* and S3*G*).

In summary, our experiments identify a class of peptidergic cells whose activation suppresses feeding behavior in adult *Drosophila*, in a manner independent of any measurable effects on metabolism or energy reserves. We suggest that AstA-expressing neurons and/or neuroendocrine cells exert an inhibitory influence on the motivation or drive to feed and/or promote aversion to unpalatable food resources. Further studies of these neurons, the circuitry they engage, and their regulation by food intake may shed light on mechanisms of satiety control and on the general question of how metabolic changes are translated into behavioral changes by the brain.

Materials and Methods

Fly Stocks and Husbandry. Flies were reared on standard media (59) at 25 °C, 70% relative humidity, under a 12 h:12 h light:dark regime unless otherwise indicated. For TRPA1 and Kir2.1 experiments, flies were raised at 19 °C and shifted to the indicated temperature 1 h before or 2 d before the experiment, respectively. Starved flies were deprived of food but not water for 24 h. Transgenic fly lines are described in *SI Materials and Methods*.

***AstA–GAL4* Transgenic Lines.** A 2,096-bp DNA fragment upstream of the putative transcriptional start site of *Drosophila AstA* (National Center for Biotechnology Information RefSeq NM_079765.2) was subcloned into pC3G4 (*Drosophila* Genomics Resource Center) (60) and microinjected into *w* embryos (BestGene).

Immunohistochemistry. Immunohistochemistry of adult male tissues was performed as described (61). Sources of antibodies and dilutions are described in *SI Materials and Methods*.

Behavioral Experiments. Feeding assays were performed using 5- to 10-d-old adult males. Nutrients were dissolved in 0.5% agar and 0.5% Food, Drug, and Cosmetic (FD&C) Blue 1, and presented as described in *SI Materials and Methods*. The fraction of flies containing blue dye after 1 h was scored under a dissecting microscope (Fig. S1). Colorimetric quantification of individual flies was performed as described in ref. 3, and the two-choice feeding assay was a slight modification of ref. 48. For PER assays (46), sucrose solutions were presented to each fly once, in order of increasing concentration. Flies responding to an initial water stimulus were discarded. Further details are provided in *SI Materials and Methods*.

Metabolic and Activity Measurements. Gut content was determined by colorimetric quantification (3) after 48 h exposure to fly food containing 0.5% FD&C Blue 1. Triglyceride and glucose levels were quantified in homogenates of flies (n = 20) using assay kits (Stanbio triglyceride kit, Sigma BCA1 protein kit, and

Sigma glucose assay kit); and see Footnote S2. Activity levels were determined using single-fly activity monitors (Trikinetics).

Data and Statistics. GraphPad Prism software was used to generate graphs and for statistical analysis. All population assays were run with all experimental and control genotypes in parallel, with at least $n = 3$ per genotype, and data are representative of multiple independent experiments. Nonparametric statistics were used in cases of unequal sample size between some genotypes, unless otherwise indicated.

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